Chem 231 Lecture No. 10 Sp 1999

SERINE PROTEASES Cont.

Last time we discussed the serine proteases, in particular details about the pH-dependence and the enzyme-substrate complexes and intermediates. **Today** we will continue and then talk a bit about site-directed mutagenesis.

The Acyl-Enzyme.

As I mention previously there is evidence that the acyl ester bond in the acyl-enzyme is distorted from the normal conformation into one resembling that from an aldehyde or ketone, i.e. one in which there is no delocalization across the C-O-Ser bond. This would presumably raise the ground state energy of the EA toward that of the TS.

Proton inventory studies on both acylation and deacylation suggest that more than one proton are involved in the rate-limiting steps. (Proton inventory is a method involving measuring the rate as a function of increasing D2O concentration). For example this may indicate that additional H-bonds are formed in the reaction.

Several X-ray diffraction studies have been done on the acyl-enzymes of non-specific substrates, stabilized at low pH, and also on an elastase EA trapped at -70oC.

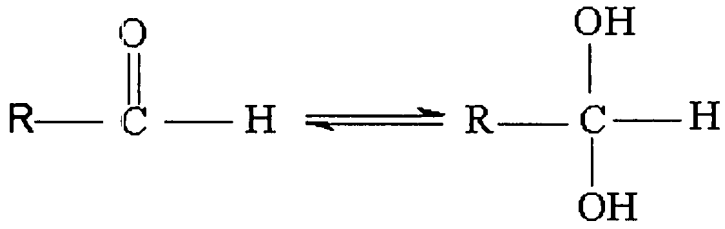
Active Site Titration.

This is the best method for determining the concentration of active enzyme and was developed by Bender with the serine proteases. The basic idea is to use a substrate which reacts very rapidly in the first part of the catalytic reaction to release a chromophoric product, e.g. rapid acylation to release p-nitrophenol, followed by a much slower reaction to complete the turnover reaction. Thus the burst of product is proportional to the number of active sites. A typical titrant for CT is cinnamoyl-imidazole, in which k1 = 1.2 x 104 M-1s-1 and k3 = 1.3 x 10-2 s-1. (Ref. JACS 88, 5890, 1966.)

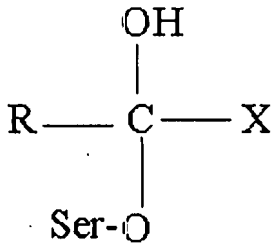
Transition State Analogs.

The idea of tight TS binding and maximum complementarity between enzyme and TS suggested to Wolfenden and Lienhard that substrate analogs which resemble the TS (at least when bound to the enzyme) should be very tight binding inhibitors. This was indeed shown to be the case for a number of enzyme/inhibitor systems. In the case of the serine proteases the three types of compounds so far shown to fit this classification are aldehyde, boronic acid and phosphonate derivatives of peptides.

The aldehydes normally exist in equilibrium with their hydrates in aqueous solution:



It is the aldehyde form, however, which is believed to bind to the enzyme, and then form a tetrahedral adduct, a hemiacetal. This is an analog of the TS:

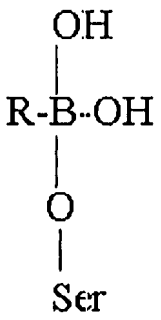


Some data for elastase illustrated this point:

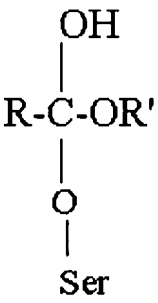
Substrate	**Ki (or Km)**
Ac-Pro-Ala-Pro-Ala-CONH2	3.9 mM
-CH2OH	0.6
-CHO	0.0008*
MeSuc- -B(OH)2	0.00000001

*The actual value is lower, since only a portion of the aldehyde is present as such, most is the hydrate.

In the case of the boronic acids the electron-hungry boron reacts with the nucleophile serine to give the tetrahedral adduct:



Transition state Analog



Transition stat

The resulting negative charge on the boron undoubtedly helps since it mimics the negative oxyanion in a real TS.

The *Thiol* Serine Proteases.

In 1968 Bender and Koshland simultaneously replaced the Ser-195 OH of subtilisin with SH, i.e. converting Ser into Cys, and a serine protease into a thiol protease. Recently it has been possible also to do the same thing with trypsin. Thiol subtilisin is active toward activated substrates (i.e. good leaving groups) but 2-3 orders of magnitude less reactive toward good substrates than subtilisin. Crystallographic and other experiments suggest no difference in the structure of the two enzymes. More recently thiol subtilisin has been made by genetic engineering (along with many other thiol replacements of active-site serines).

Several theories have been proposed to account for the decreased reactivity of thiol subtilisin. It probably arises in part

from the better partitioning of TI back to starting material (compare the pK's of CysSH and Ser OH to the leaving groups), and the lesser nucleophilic character of S which necessitates prior protonation of the leaving group for formation of the TI.

OTHER CLASSES OF PROTEASE

	Active site residue	Mechanism	Examples
Serine proteases	Ser	Covalent EA	Trypsin, subtilisin
Thiol proteases	Cys	Covalent EA	Papain, ficin, bromelain
Aspartyl (acid) proteases	Asp (two)	Non-covalent, g.a./g.b.	Pepsin, HIV protease
Metallo proteases	Zn^+	Electrophilic	Thermolysin, carboxypeptidas

CHEMICAL MODIFICATION OF ENZYMES.

We will take a brief look at how one can use structural modification of enzymes to learn about functional aspects. There are two major approaches, chemical modification and genetic modification, via site-directed mutagenesis. We will first look at "crude" methods, namely residue-specific reagents to label potential essential catalytic groups, then refined methods using affinity labels and active-site-directed modifying reagents, and finally we will briefly go over site-directed mutagenesis methods to make specific changes in amino acid residues, to probe their function.

The methods I am going to talk about today are ones which are used to determine which amino acids are important for the catalytic function of an enzyme. Such information is obviously essential if one is going to propose a reasonable mechanism for the catalytic reaction and to explain the relationship between the structure of the protein and its function. It is also important in drug design.

The most common groups found in active sites are:

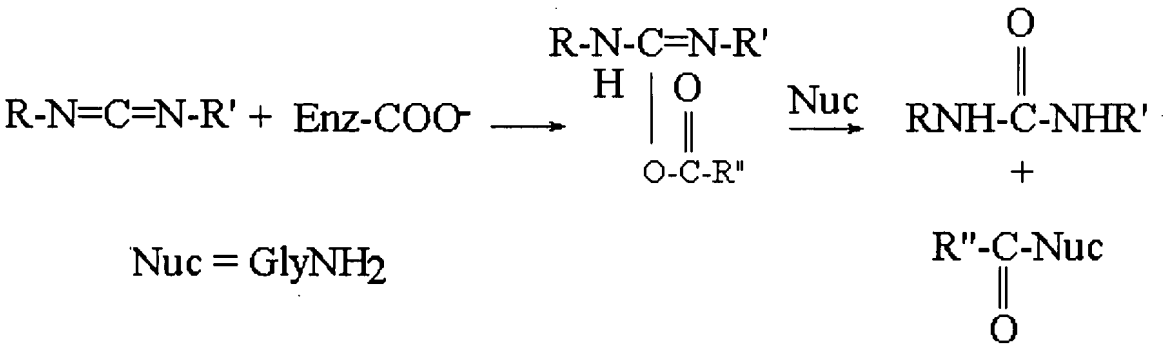
His	Lys	Arg	Tyr
Asp	Glu	Cys	Ser

Chemical modification of AA residues.

The use of residue-specific reagents to probe for essential residues is an ancient one. The principle is straightforward, but the method suffers from a number of drawbacks, most particularly the lack of true specificity of most reagents. Fersht lists some common such reagents in a Table.

For example, suppose one wants to determine if a carboxylate is essential. One might react the enzyme with Water-Soluble-Carbodiimides, such as EDAC. (Carbodiimides are: R-N=C=N-R')

The activated carboxyls are blocked with a small nucleophilic molecule, frequently glycine amide.



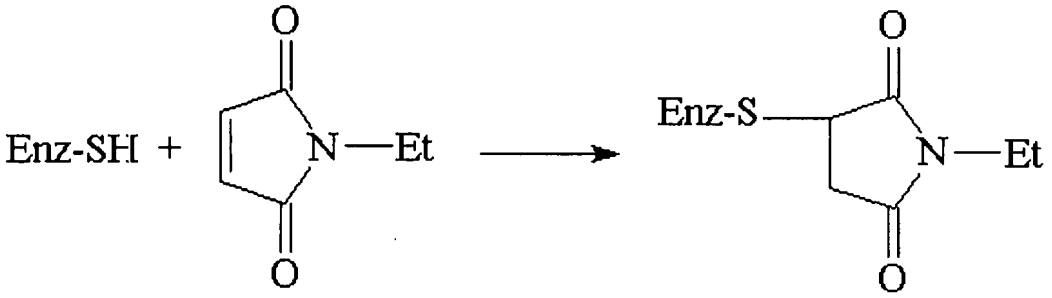
Other examples are: Aldehydes plus BH_4^- for amines: Enz-NH_2 + RCHO → Enz-N=CHR

+ BH^-_4 → EnzNH-CH_2R

Also methyl imidates for amines: Enz-NH_2 + CH_3O-C(=NH)-R → Enz-NH^+=C(NH_2)R

(Di-aldehydes and di-imidates are often used for cross-linking - via amines: most common is glutaraldehyde)

Cysteines are often detected with iodoacetate or iodoacetamide, or NEM, N-ethyl maleimide:



Histidines are detected with diethylpyrocarbonate: EtOC(=O)-O-C(=O)-OEt

Substrate or inhibitor protection is also often used. The method often yields false information (e.g. β -lactamases).

Active-site directed modifying reagents or affinity labels.

These are an attempt to bring specificity to side-chain modification. The basic idea is to combine a fragment of the substrate with a reactive functional group. The former builds in specificity to the active-site (sort of the magic bullet part) while the latter causes covalent modification of appropriate groups to occur. The classic case involves trypsin and TLCK (tosyl-Lys-chloromethyl ketone) which closely resembles the substrate Ts-Lys-OMe (which we already discussed). The activated ketone is susceptible to nucleophilic attack and is in fact attacked by the imidazole of the active-site His.

Another example is ATP which has been activated by periodate oxidation so that the ribose contains two aldehyde groups. These can react with amines, to yield imines which can be stabilized by reduction with cyanoborohydride.

PMSF (phenylmethanesulfonyl fluoride), commonly used to inhibit proteases is an active-site modifying reagent:

Photoaffinity labels have also been used. The basis in these cases is to incorporate a light-activated reactive group,

typically an azo derivative to generate a nitrene (-N:) or a carbene (=C:); these are highly reactive, short-lived species which insert into nearby bonds.

(See transparencies for examples)